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International Journal of Pharmaceutics 170 (1998) 73–84

international journal of pharmaceutics

Development of a dispersal procedure for the lipid A analog E5531 using a 'pH-jump method'

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Received 21 January 1998; received in revised form 6 March 1998; accepted 18 March 1998

Abstract

We have developed a 'pH-jump method' in order to facilitate the dispersion of E5531, a synthetic analog of lipid A, at neutral pH for use in pharmaceutical preparations. The 'pH-jump method' procedure involves dispersing E5531 at pH 11.0 (above pK_2) at 50°C (higher than the phase transition temperature) followed by mixing with phosphate buffer in order to adjust the solution to a neutral pH. The size of the aggregates prepared by the pH-jump method was approximately 15 nm. However, when E5531 was dispersed in neutral pH (7.3), directly (normal dilution method), the size of aggregates was approximately 120 nm and when sonication was used (sonication method), the size of the aggregates was approximately 45 nm and larger than that prepared by pH-jump method. E5531 aggregates form vesicle structures. The membrane fluidity and micropolarity of the aggregates prepared by the pH-jump method was higher than that of those prepared by the normal dilution and sonication methods. This study therefore shows that the pH-jump method will provide smaller, fully hydrated aggregates of E5531 than either the normal dilution or sonication methods.

Keywords: Lipid A analog; pH-Jump; Heating; Dispersing; Hydration

1. Introduction

Lipid A is a component of bacterial lipopolysaccharides (LPS), which are present in the major amphiphilic constituents of the leaflet of Gram-negative bacteria. Lipid A is a potent biological active site (Galanos et al., 1977; Morrinson and Ryan, 1979) and induces the prostaglandins, cytokines such as interferon (Homma et al., 1985) interleukin 1 (Koide and Steinman, 1987) and tumor necrosis factor (TNF; Beutler and Cerami, 1986) in mammalian cells such as macrophages and lymphocytes. This compound also induces undesirable toxic effects such as fever and the Schwartzmann bleeding reaction (Vogel et al., 1984; Galanos et al., 1985).

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0378-5173/98/$19.00
PII S0378-5173(98)00131-8

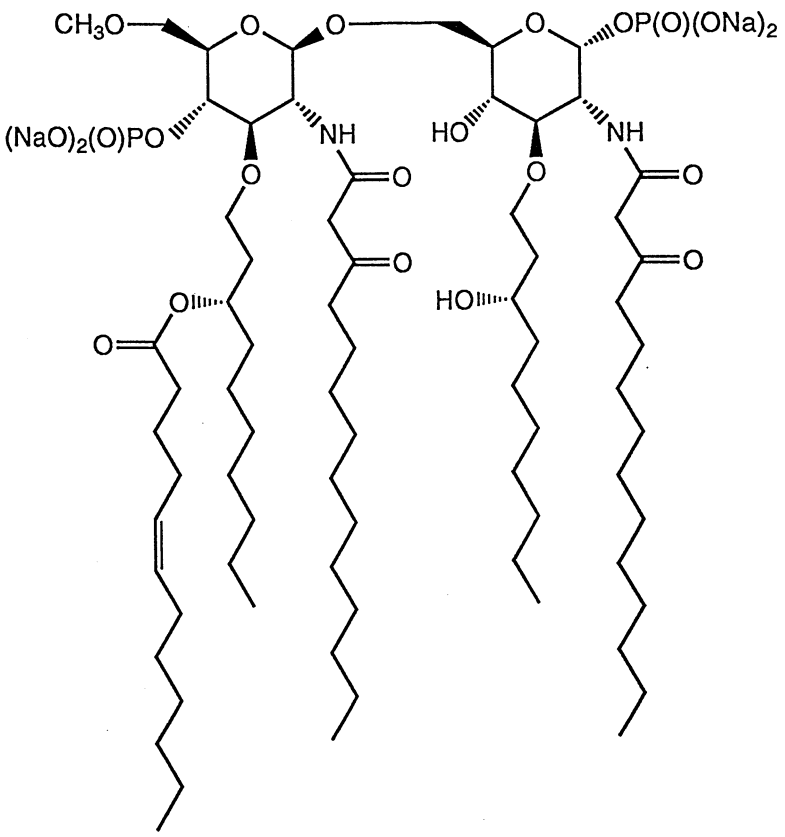

Fig. 1. Chemical structure of the synthetic lipid A analog E5531.

Many attempts have been made to synthesize low toxicity lipid A analogs. Christ et al. (1995) have indicated that E5531, a synthetic disaccharide analog of lipid A (Fig. 1), has low toxicity and retains some of the biological activities of lipid A such as the reduction in TNF production. This compound has been found to be a specific LPS antagonist in an LPS-binding assay and is an inhibitor of LPS-induced TNF production in monocytes/macrophages, so it is therefore possible that E5531 will be useful for the treatment of septic shock.

An injectable E5531 formulation would be extremely useful, however, the dispersion of E5531 in aqueous solution has been a major problem. E5531, like many lipid A analogs, does not disperse well in solutions with a neutral pH. Sonication has been used to disperse lipid A analogs (Labischinski et al., 1990; Hofer et al., 1991) and LPS (Alphen et al., 1980) for investigational use. However, sonication has two major drawbacks—it is not suitable for large scale production and it is difficult to control the sonication energy and dispersal time (Huang, 1969; Johnson et al., 1971). Ideally, the pH of E5531 pharmaceutical injections should be neutral since the injection of alkaline or acidic pH solutions is undesirable. It is also important to reduce the size of the aggregates to obtain a more transparent solution because the size of colloidal particles is correlated with their hepatic uptake and a smaller aggregates size (less than 100 nm) may result in reduced hepatic uptake (Sezaki, 1986).

In order to overcome these problems, we have developed a new 'pH-jump method' for dispersing E5531, the advantages of this method include its suitability for large scale production (without any mechanical input such as sonication) and the

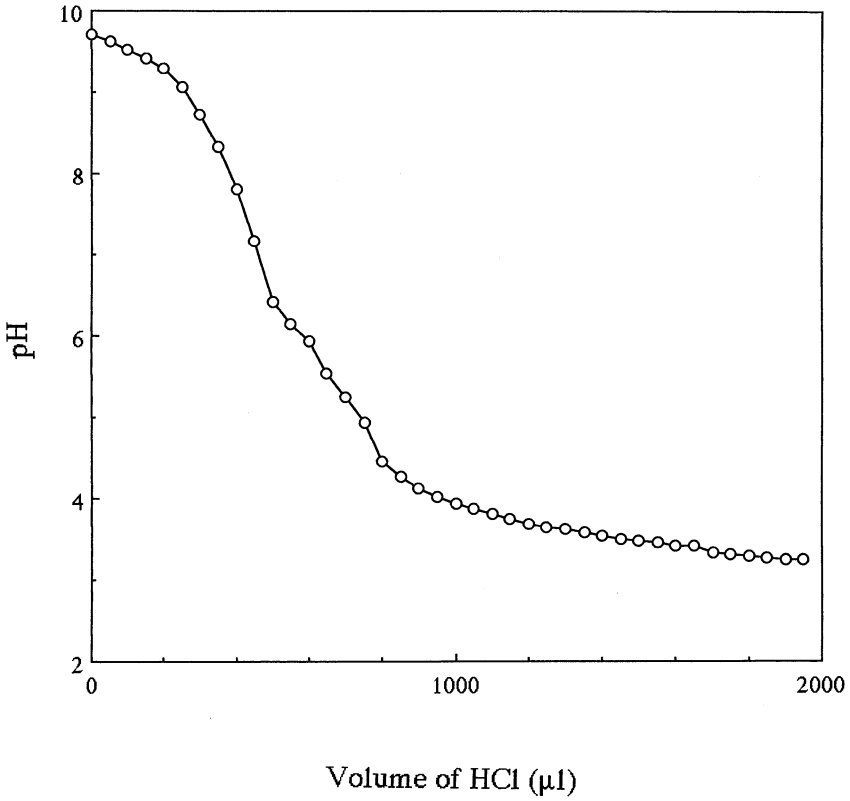

Fig. 2. pH titration curve for E5531. A 0.2-mM solution of E5531 was titrated with 0.2 mM HCl. The pH was recorded as a function of the added volume of HCl. $pK_1 = 6.0$ and $pK_2 = 9.3$.

preparation of smaller sized of E5531 aggregates (approximately 15 nm) at neutral pH. In addition, in order to clarify the behavior of E5531 aggregates in neutral pH (pH 7.3), various physicochemical properties such as the size of the aggregates, the trapped volume of molecular assemblies, membrane fluidity and micropolarity were measured and compared with those prepared by the normal dilution method which was dispersed in neutral pH (7.3), directly or was dispersed by sonication.

2. Materials and methods

2.1. Materials

E5531 was obtained from Eisai Chemical (Ibaraki, Japan). Calcein (3,3′-bis [*N*,*N*-bis (carboxymethyl) aminomethyl]-fluorescein) was supplied by Dojin (Kumamoto, Japan). *N*-dansylhexadecylamine (DSHA) was from Lambda (Graz, Austria) and 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Tokyo-Kasei (Tokyo, Japan). Lactose hydrous, sodium phosphate monobasic, sodium phosphate dibasic and sodium hydroxide were supplied by Mallinckrodt (Paris, KT).

2.2. Methods

2.2.1. Determination of pK_a

Three milligrams of the tetra sodium salt of E5531 were added to 8 ml of water and the mixture was dispersed by sonication for 3 min at 50°C. A probe-type sonicator was used (Model UR-200P, Tomy Seiko Company Ltd., power setting: 100 W). The solution was cooled to room

temperature and the final volume adjusted to 10 ml with water (0.2 mM E5531). The pK_a and the dissociated form of E5531 were determined by titrating the solution with 0.2 mM HCl.

2.2.2. Determination of the phase transition temperature

In order to determine the phase transition temperature of E5531, differential scanning calorimetry (DSC) of E5531 was performed using a Model DSC-100 (Seiko-Denshi, Tokyo, Japan). Ten milligrams of E5531 in 40 μl of 0.003 N NaOH solution (pH 11.0) or 40 μl of 4.25 mM phosphate buffer containing 10% lactose solution (pH 7.3) were placed in a DSC pan and sealed. An equal volume of 0.003 N NaOH solution or the phosphate buffer was placed in the reference pan. Temperature scans were made from 10 to 70°C with a heating rate of 1.25°C/min. All calorimetric data were obtained from samples during the heating phase. Molar enthalpies were obtained from the molar concentration of E5531.

2.2.3. Comparison of the size of the E5531 aggregates prepared using the pH-jump, normal dilution and sonication method

The size of the E5531 aggregates prepared using the 'pH-jump' method, 'normal dilution' and sonication methods were measured. The used procedures were as follows.

2.2.3.1. pH-jump method (pH 11.0→pH 7.3). Thirty milligrams of E5531 were dispersed in 15 ml of 0.003 N NaOH solution (pH 11.0) with stirring at 50°C. After stirring for 10, 20, 30, 40 and 60 min, 1.5 ml of the alkaline solution were sampled and mixed with phosphate-NaOH buffer containing lactose. The sample volume was adjusted to 10 ml by adding water to the formulated solution (E5531: 300 μg/ml, 4.25 mM phosphate-NaOH buffer containing 10% lactose solution, pH 7.3). The size of the aggregates was measured at 25°C by the dynamic light scattering (DLS) technique using a laser particle analyzer equipped an Argon laser (Model DLS-7000DL, Ohtsuka Electronics, Osaka, Japan). The data were analyzed by the histogram method (Gulari et al., 1979) and the weight-averaged size of the aggregates was evaluated.

2.2.3.2. Normal dilution method (pH 7.3→pH 7.3). Thirty milligrams of E5531 were dispersed in 15 ml of buffer solution (4.25 mM phosphate-NaOH buffer containing 10% lactose, pH 7.3) with stirring at 50°C. After stirring for 10, 20, 30, 40 and 60 min, 1.5 ml of the solution were sampled and diluted with the phosphate-NaOH buffer containing lactose. The volume was adjusted to 10 ml by adding the buffer solution (E5531: 300 μg/ml, 4.25 mM phosphate-NaOH buffer containing 10% lactose solution, pH 7.3). The size of the aggregates was measured using the procedure outline above.

2.2.3.3. Sonication method (pH 7.3→pH 7.3). Thirty milligrams of E5531 were dispersed in 15 ml of buffer solution (4.25 mM phosphate-NaOH buffer containing 10% lactose, pH 7.3) with sonication at 50°C using a probe-type sonicator (Model UR-200P, Tomy Seiko Co., Ltd., Power setting: 100 W). After sonication for 10, 20, 30, 40 and 60 min, 1.5 ml of the solution was sampled and diluted with the phosphate-NaOH buffer containing lactose. The volume was adjusted to 10 ml by adding the buffer solution (E5531: 300 μg/ml, 4.25 mM phosphate-NaOH buffer containing 10% lactose solution, pH 7.3). The size of the aggregates size was measured using the procedure outline above.

2.2.4. Determination of trapped volume

In order to obtain an information on the structure of the E5531 aggregates, the trapped volume inside the aggregates was determined. E5531 was dispersed in a 70 mM calcein solution using the pH-jump, normal dilution and sonication method, respectively. The procedures were as follows.

2.2.4.1. pH-jump method. Five milligrams of E5531 was dispersed in 2.5 ml of 70 mM calcein solution (pH 11.0) with stirring at 50°C for 60 min. After cooling to 25°C, the pH of the solution was adjusted to 7.3 by the addition of 1 N HCl solution.

2.2.4.2. Normal dilution method. Five milligrams of E5531 was dispersed in 2.5 ml of 70 mM calcein solution (pH 7.3) with stirring at 50°C for 60 min and then cooled to 25°C.

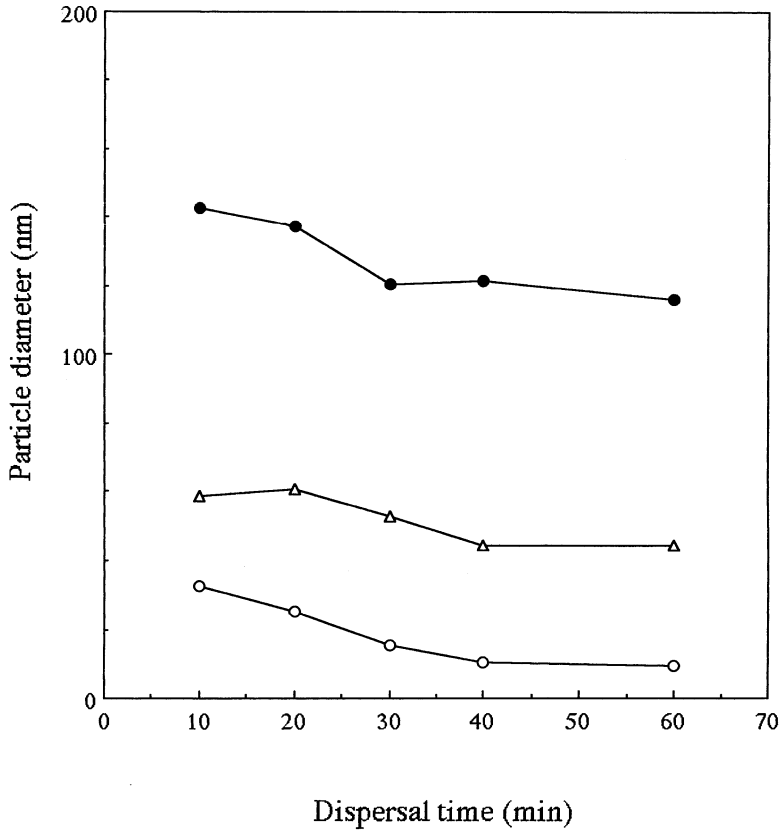

Fig. 3. Relationship between dispersal time and the size of the E5531 aggregates evaluated by the dynamic light scattering (DLS) methods (in weight). The polarization of E5531 aggregates prepared by pH-jump method (○– – –○), normal dilution method (●– – –●) and sonication method (△– – –△).

2.2.4.3. Sonication method. Five milligrams of E5531 was dispersed in 2.5 ml of 70 mM calcein solution (pH 7.3) with sonication at 50°C for 60 min and then cooled to 25°C.

The untrapped calcein was removed by gel filtration (Sephadex G-50) at 25°C. The volume of the calcein solution trapped in the dispersed aggregates was determined fluorometrically (Allen and Cleland, 1980) after solubilization of the lipid aggregates by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of E5531 was evaluated. E5531 in the dispersion was assayed by HPLC method (Detection wavelength: 254 nm).

2.2.5. Determination of the membrane fluidity of E5531 aggregates prepared by the pH-jump, normal dilution and sonication method

In order to compare the membrane fluidity of E5531 aggregates prepared by the pH-jump, normal dilution and sonication method, fluorescence polarization techniques (Probe: DPH) as reported by Shinizky (1984) were used. Membrane fluidity of E5531 formulated solution using pH-jump method (stirring for 60 min), normal dilution method (stirring for 60 min) and sonication method (for 60 min) were evaluated from 15 to 50°C. A solution of DPH in tetrahydrofuran was added to E5531 solution at a final concentration

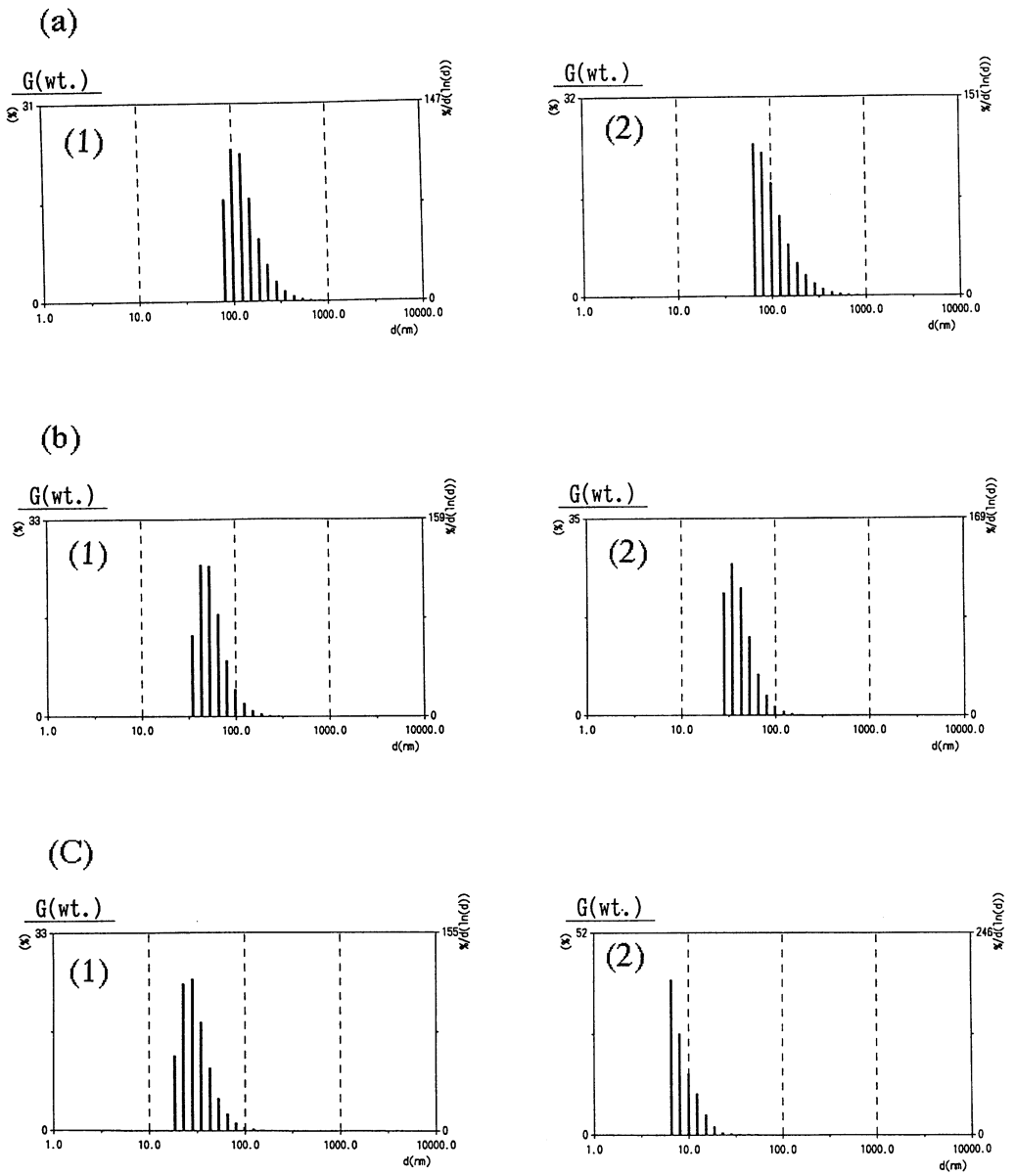

Fig. 4. Histograms for size distribution of the dispersed particles evaluated by the dynamic light scattering (DLS) methods (in weight). (a): E5531 aggregates prepared by the normal dilution method: (1) Dispersed for 10 min. Mean diameter: 142.9 ± 78.2 (nm); (2) Dispersed for 60 min. Mean diameter 116.9 ± 77.3 (nm). (b): E5531 aggregates prepared by the sonication method: (1) Dispersed for 10 min. Mean diameter: 59.0 ± 25.9 (nm); (2) Dispersed for 60 min. Mean diameter: 44.2 ± 19.2 (nm). (c): E5531 aggregates prepared by the pH-jump method: (1) Dispersed for 10 min. Mean diameter 32.6 ± 15.0 (nm); (2) Dispersed for 60 min. Mean diameter: 9.2 ± 5.3 (nm).

of 0.5 μM. The final concentration of tetrahydrofuran added was 0.1% to avoid perturbation of the membrane. DPH was added at 1 mol% to total lipids. E5531 suspensions were incubated with DPH for 60 min at 50°C. Fluorescence polarization was measured with a H-4500 spectrometer

(Hitachi, Tokyo, Japan). The excitation and emission wavelengths used for DPH were 360 and 428 nm, respectively. The degree of polarization (P) was defined by Eq. (1):

$$P = (I_{VV} - C_f I_{VH})/(I_{VV} + C_f I_{VH}) \quad (1)$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analyze (second) polarizers, respectively. $C_f = (I_{HV}/I_{HH})$ is the grating correction factor.

2.2.6. Determination of the micropolarity of E5531 membranes

In order to determine the degree of hydration, the micropolarity of the E5531 aggregates prepared by the above three methods was determined using a fluorescence techniques (Probe: DSHA, Waggoner and Stryer, 1970). DSHA has been reported to provide information on the phase transition due to observed large increase in fluorescence intensity, mainly due to a higher partitioning of dye in the head group phase of fluid bilayers (Iwamoto and Sunamoto, 1981). DSHA was added at a concentration 1 mol% of the total lipids. The fluorescence spectra were measured upon excitation at 330 nm. All fluorescence measurements were carried out using a Model F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The micropolarity around DSHA incorporated into the E5531 aggregates was evaluated using the wavelength of maximum intensity of emission. DSHA (3.1 mg) was dissolved in 10 ml of methanol, ethanol, propanol, butanol, acetone, tetrahydrofuran, benzene and hexane, respectively. Five microliters of each solution were then diluted with 5 ml of the same solvent. The wavelengths at the maximum fluorescence intensity of each solvent were plotted against the polarity of each solvent (Dimorth and Reichardt, 1963).

3. Results and Discussion

3.1. Determination of pK_a

Fig. 2 represents the titration curve for the E5531 dispersion (0.2 mM) with 0.2 mM HCl and shows two pK values, at approximately 6.0 (pK_1) and 9.3 (pK_2), respectively. At neutral pH, E5531 existed almost entirely as the big-salt (2-Na) form. At a pH of 10 or above in aqueous solution, E5531 was almost completely present in the dissociated form. This result suggested that it would be difficult to disperse E5531 at neutral pH but that it would be easily in alkaline solution, especially above pH 10.

3.2. Determination of the phase transition temperature of E5531 using DSC

It is well known that lipids such as phospholipids have a phase transition temperature. Dipalmitoylphosphatidylcholine (DPPC) has a phase transition temperature of 41°C and dimylstoylphosphatidylcholine (DMPC) of 23°C (Marbrey and Sturtevant, 1978). Above the phase transition temperature, hydration is accelerated and dispersal is easier than at below the phase transition temperature. We examined the phase transition temperature of E5531 at both pH 7.3 and 11.0.

DSC measurements revealed that the phase transition temperatures of E5531 in 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3) and in 0.003 N NaOH solution (pH 11.0) were 31.7 and 31.6°C, respectively. Based upon the results, it is expected that hydration will be accelerated above the phase transition temperature and we decided on a dispersal temperature of 50°C and evaluated the effect of pH at 11.0 and 7.3 on the dispersal of E5531. In addition, we

Table 1
Particle diameter evaluated by dynamic light scattering (DLS) and trapped volume of E5531 aggregates prepared by normal dilution, sonication and pH-jump method

	Particle diameter (nm)	Trapped volume (l/mol)
E5531 aggregates prepared by normal dilution	128.9 ± 75.3	0.91
E5531 aggregates prepared by sonication	47.1 ± 19.9	0.66
E5531 aggregates prepared by the pH-jump	13.1 ± 4.4	0.27

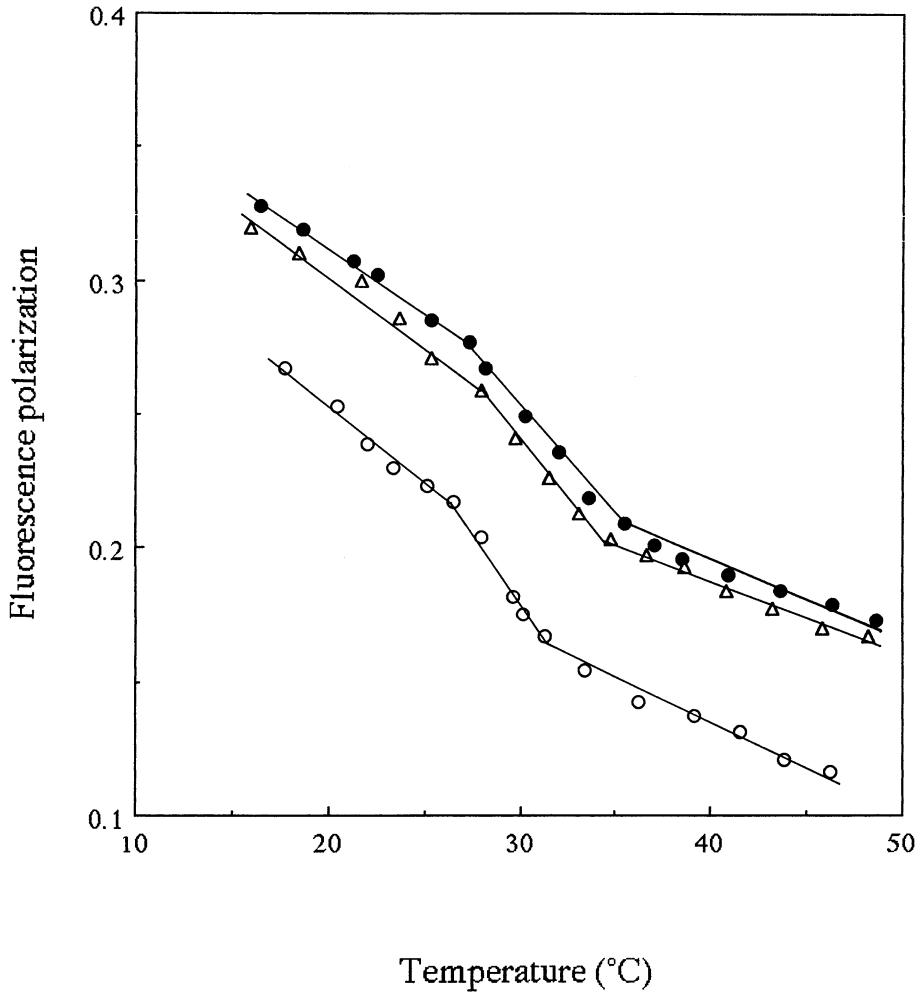

Fig. 5. Relationship between temperature and fluorescence polarization of E5531 aggregates determined using a fluorescence probe, DPH. Polarization of E5531 aggregates prepared by pH-jump method (○– – –○), normal dilution method (●– – –●) and sonication method (△– – –△).

examined the effect of sonication on the dispersal of E5531. We examined the three preparation procedures (pH-jump, normal dilution and sonication method) by monitoring the size of the E5531 aggregates during dispersing.

3.3. Comparison of the size of E5531 aggregates prepared by the pH-jump, normal dilution and sonication method

Fig. 3 represents the relationship between dispersal time and the size of the E5531 aggregates prepared by the pH-jump, normal dilution and sonication methods. The size of the E5531 aggregates prepared by the normal dilution, sonication and pH-jump methods after a 10 min dispersal period were 140, 60 and 35 nm, respectively. The size decreased with increasing dispersal time and reached constant values of approximately 120, 45 and 15 nm, respectively after 40 min. The size of the E5531 aggregates prepared by the pH-jump method after a 60 min dispersal period was also examined by electron microscopy. This method gave a size of 17 nm for the E5531 aggregates,

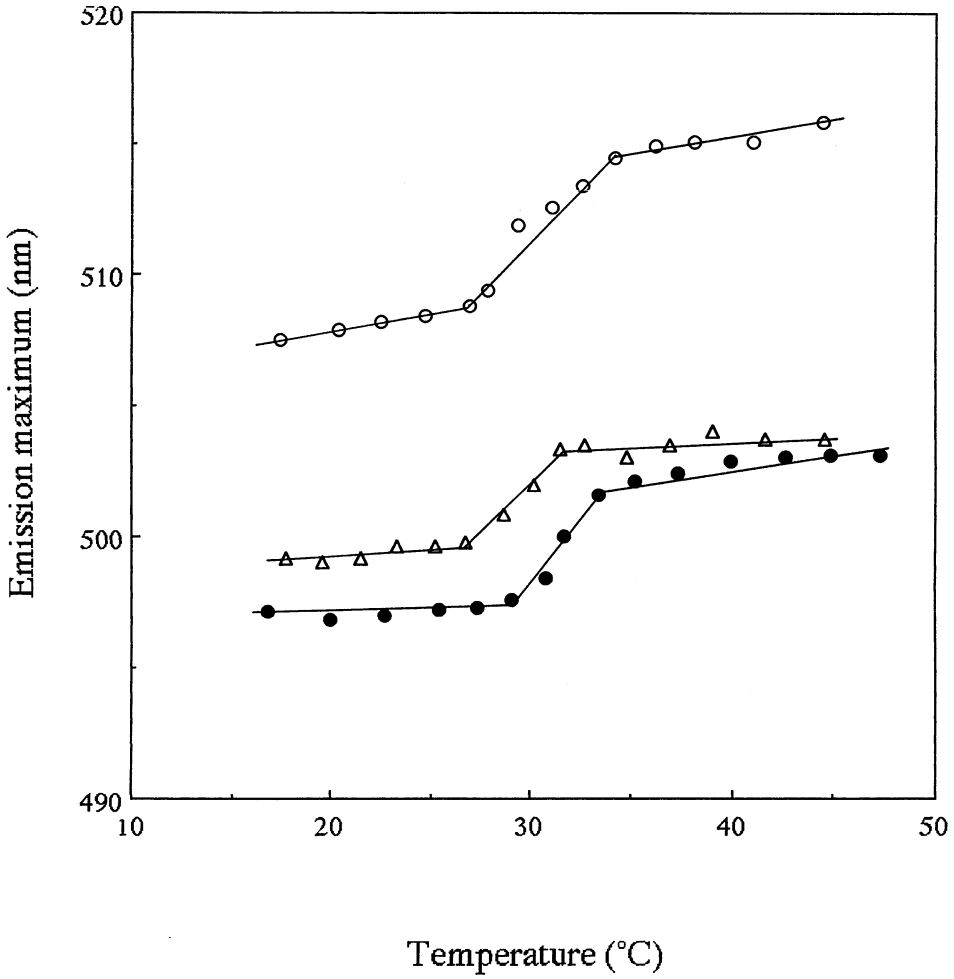

Fig. 6. Relationship between temperature and emission maximum of E5531 aggregates using a fluorescence probe, DSHA. The emission maximum of E5531 aggregates prepared by pH-jump method (○– – –○), normal dilution method (●– – –●) and sonication method (△– – –△).

similar to the result obtained with the DLS method.

Fig. 4 represents the DLS histograms for E5531 aggregates prepared by the pH-jump, normal dilution and sonication methods after dispersing for 10 and 60 min. The size distribution of the E5531 aggregates prepared by normal dilution and sonication were similar after dispersal times of 10 and 60 min. However, the size of the aggregates prepared by the pH-jump method after dispersal for 60 min was smaller than that after 10 min and the mean diameter had decreased from 35 to 15 nm.

At a neutral pH, E5531 exists as a bis sodium salt and E5531 molecules are stabilized by the hydrogen bonds of the head group. It has been reported that intermolecular hydrogen bonding has been suggested to affect both the phase state and overall order of state in acidic phospholipid (Eibl, 1983). Structural stabilization of phosphatidic acid aggregates is the greatest when the phosphates are semi-ionized (Brandenbrug and Seydel, 1984). At basic pH values above pK_2 (9.3), E5531 is fully ionized and exists as the dissociated form and hydration can be accelerated with the loss of hydrogen bonds in the head group.

Sonication has been used in order to obtain small unilamella vesicles of phospholipids (Huang and Mason, 1978). The size of E5531 aggregates was decreased to 45 nm by sonication. However, by dispersing E5531 in an alkaline solution above pK_2 (9.3) and above the phase transition temperature (around 30°C), we have successfully obtained a smaller aggregates sizes (approximately 15 nm) without an energy input such as sonication. Therefore, the advantage of the pH-jump method for preparing formulations of E5531 is the suitability of this method for large scale production.

3.4. Structure of E5531 aggregates prepared by the pH-jump, normal dilution and sonication methods

Table 1 shows the volumes of trapped inner space in the aggregates per mole of E5531 prepared by the pH-jump, normal dilution and sonication methods. The trapped volumes of small unilamellar vesicles (diameter: 20–50 nm), large unilamellar vesicles (200–1000 nm) and multilamellar vesicles (diameter: 400–3500 nm) of phosphatidylcholine have been estimated to be 0.2–0.5, 3–4 and 7–10 l/mole, respectively (Szoka and Papahadjopoulos, 1978). E5531 aggregates prepared by the pH-jump method (diameter: 13.1 nm) had a trapped volume of 0.27 liters per mole. This data indicates that E5531 molecules prepared by this method have liposome-like structures (small unilamellar vesicles). E5531 aggregates prepared by the normal dilution method (diameter 128.9 nm) and sonication method (diameter: 47.1 nm) had a trapped volume of 0.91 and 0.66 l/mole, respectively. These data indicates that the structures of these aggregates are origolamella vesicles.

3.5. Membrane fluidity of E5531 aggregates

The membrane fluidity of the E5531 aggregates was determined by a fluorescence polarization method using DPH as the hydrophobic fluorescence probe. Fig. 5 presents a plot of fluorescence polarization versus temperature for E5531 aggregates prepared by the pH-jump, normal dilution and sonication methods to evaluate the membrane fluidity. From this plot, the phase transition temperatures can be estimated (approximately 30°C), respectively. Fluorescence polarization of E5531 aggregates prepared by pH-jump method is the smallest among them and those prepared by the normal dilution and sonication methods were larger and similar. This indicates that the membrane fluidity of the aggregates prepared by the pH-jump method is the largest and that prepared by normal dilution method is the smallest. Using sonication, the size of the aggregates (45 nm) was smaller than those prepared by the normal dilution method (120 nm), however, the membrane fluidity of the aggregates prepared by each method was similar. Therefore, while sonication was shown to reduce the size of the aggregates, it did not affect the membrane fluidity of the aggregates.

3.6. Micropolarity of E5531 aggregates

The micropolarity of the E5531 membrane was determined. The emission maxima of DSHA embedded in E5531 aggregates prepared by the pH-jump, normal dilution and sonication methods were determined as a function of incubation temperature (Fig. 6). It has been reported that the fluorescence characteristics of DSHA depend on the micropolarity around the probe and that the dansyl fluorophore is located in the glycerol backbone of liposomal bilayers (Iwamoto and Sunamoto, 1981). It has also been reported that hydration increased greatly in phospholipid liposomes above the phase transition temperature (Iwamoto et al., 1982). Therefore, it is expected that the emission maxima of DSHA in E5531 aggregates will provide information on the micropolarity around their surface. The emission maxima for E5531 aggregates prepared by the pH-jump, normal dilution and sonication method at 25°C were approximately 508.3, 497.1 and 499.5 nm, respectively, indicating that the micropolarity around the probe in E5531 aggregates is comparable to that of butanol, acetone and acetone, respectively. This indicates that the

degree of hydration for E5531 aggregates prepared by the normal dilution and sonication method is similar but lower than that of aggregates prepared by the pH-jump method. In other words, with a sonication, the aggregates size is reduced from 120 (normal dilution method) to 45 nm, sonication will not affect the degree of hydration in the aggregates and only reduce the size.

Above the phase transition temperature the maximum wavelengths increased and exhibited a red shift, indicating that the micropolarity around the surface of E5531 aggregates increased and hydration was accelerated above the phase transition temperature. The phase transition temperature was confirmed to be about 30°C in this micropolarity study and this finding is coincident with DSC and fluorescence polarization results.

4. Conclusions

E5531 was dispersed using a novel pH-jump method, which involves dispersing E5531 in an alkaline solution (0.003 N NaOH, pH 11.0, above $pK_2 = 9.3$) at 50°C (higher than the phase transition temperature of approximately 30°C) and then mixing the solution with the buffer to neutralize the pH to 7.3. The size of aggregates obtained using this method was approximately 15 nm and smaller than that of those prepared by either normal dilution (120 nm) or sonication method (45 nm). E5531 aggregates form vesicle like structures. The membrane fluidity and micropolarity of the aggregates prepared by the pH-jump method was larger than those prepared by the normal dilution and sonication methods. In other words, small fully hydrated E5531 aggregates can be produced by the pH-jump method, but not by the normal dilution or sonication method.

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